REMARKS

Applicants respectfully request reconsideration of this application, and reconsideration of the Office Action dated November 17, 2004. Upon entry of this Amendment, claims 33, 34, 36, 38, and 40-97 will remain pending in this application with claims 42-54, 60, 61, 63, 68, 69, 71, 72, 75-87 and 90-97 being withdrawn. The changes to the claims are fully supported by the specification and original claims. No new matter is incorporated by this Amendment.

* * * * *

Claims 73 and 74 are rejected under 35 U.S.C. § 112, first paragraph, for purportedly failing to provide an enabling disclosure. Specifically, the Office Action asserts the specification does not reasonably provide enablement for diagnosis and treatment of every disease. Applicants respectfully traverse.

Applicants point out that the specification is not required to teach that which is known to those of skill in the art. Moreover, "[a]n applicant is not required to describe in the specification every conceivable and possible future embodiment of his invention." *Rexnord Corp. v. Laitram Corp.*, 274 F.3d 1336, 1344 [60 USPQ2d 1851] (Fed. Cir. 2001). In addition, "a specification may, within the meaning of 35 U.S.C. § 112 para. 1, contain a written description of a broadly claimed invention without describing all species that [the] claim encompasses." *Utter v. Hiraga*, 845 F.2d 993, 998 [6 USPQ2d 1709] (Fed. Cir. 1998). However, even with the above case law in mind, Applicants submit that the specification more than adequately teaches how to make and use the claimed invention.

The specification adequately describes how to make the claimed reagent. Moreover, the specification, at pages 2 and 3, teaches that known compounds can be incorporated into the tri-functional reagent and the resulting reagent can be used to treat or diagnose disease. In other words, the specification provides numerous examples of compounds that are known to treat or diagnose diseases in mammals. In addition, there are

numerous reagents known to those of ordinary skill in the art which can be used to treat and diagnose disease. Moreover, these known reagents can be improved by incorporating them into Applicants' new tri-functional reagent of the present invention.

With all due respect, Applicants would gently remind the Examiner that the basis for raising the enablement statute is for instances where the specification fails to provide sufficient guidance to one of ordinary skill in the art to practice the invention within the scope of the claims without undue experimentation. There are demonstrative examples to guide the skilled practitioner through the various elements to make and use the claimed reagent.

The Office Action has raised no substantial evidence or analysis to challenge the presumption of enablement. To maintain this rejection, the Office Action must disclose persuasive evidence with a detailed analysis to demonstrate the reasoning as to why the specification would not enable one of ordinary skill in the art to practice the invention without undue experimentation.

There is clearly sufficient disclosure in the specification as to how the claimed reagent may be practiced. It is reasonable to conclude that the specification does teach how to practice the invention according to the full extent claimed. The Office Action has not set forth a reasonable explanation why the rejected claims are not enabled by the specification and accordingly, Applicant respectfully traverses the rejection and requests that it be withdrawn.

* * *

Claims 70 and 98 are rejected under 35 U.S.C. § 112, second paragraph, as indefinite.

Claim 98 has been canceled and claim 70 has been amended to only recite reagent 45. Hence, the rejection is overcome and it withdrawal is respectfully requested.

* * *

* * *

Claims 33, 34, 40, 41, 55-59, 62, 64-67, 70, 73, 74, 88, and 89 are rejected under 35 U.S.C. § 103(a) as purportedly obvious based on Wilbur et al. (WO 97/29114) in view of Rosebrough (J. Pharm. Exp. Ther., 1993). Applicants traverse this rejection.

Independent claim 33 (from which the other claims depend) concerns a single molecule reagent for conjugation to a biomolecule, provided with at least three functional parts, and corresponding to a specifically recited schematic structure. The reagent includes an affinity ligand, coupled to the trifunctional cross-linking moiety via a first linker and a biotinamide bond which has been stabilized to inhibit enzymatic cleavage. Stabilization is accomplished by introducing an aspartyl moiety in linker 1 of the affinity ligand such that binding with avidin or streptavidin is not diminished by sterical hindrance.

The Office Action concedes that neither Wilbur nor Rosebrough discloses the use of an aspartyl moiety to inhibit enzymatic cleavage. However, the Office Action asserts that since Rosebrough teaches employing introducing a carboxyl group alpha to an amide bond, it would have been obvious to introduce a carboxyl group beta to an amide bond. The Office Action then concludes that combined teachings of the two references renders the claimed invention obvious. Applicants respectfully disagree for the following reasons.

The Office Action correctly points out that Rosebrough teaches pharmacokinetics, and in vitro and in vivo stability (of a series) of radiolabeled deferoxamine-biotin derivatives in plasma. However, the conclusion that "Rosebrough teaches that introduction of a carboxyl group alpha to the amide bond of biotinamide, blocks the biotinidase activity, thereby increasing the stability of biotinamide bond towards enzymatic cleavage and also the binding ability towards avidin in vivo and in vitro" is incorrect. At the time of the invention was made, it was not obvious to use an aspartyl moiety to block biotinidase.

The paper by Rosebrough (*Journal of Pharmacology Experimental Therapeutics 265*, 408-415, 1993) does not teach one skilled in the art that conjugation of biotin with cysteine will provide complete blockage of biotinamide cleavage by the enzyme biotinidase when that conjugate is coupled with another molecule. The paper provides data that indicates a biotin directly coupled with deferoxamine (DB) is stabilized from biotinidase cleavage (45% remaining after 24h) and that the combination of biotin, cysteine and deferoxamine further blocks biotinidase activity (87% remaining after 24h). The data does not provide information on whether biotinidase is blocked when biotin and cysteine are coupled with a molecule other than deferoxamine. Further, the data indicate that the blockage is only 87% at 24h. While this data suggest that an alpha carboxylate group might block biotinidase activity, it does not prove it and a person skilled in the art could not be assured that was the case. Moreover, one of ordinary skill in the art after reading Rosebrough would not have found it obvious that an aspartyl moiety would block biotinidase.

In the Office Action, it is asserted that "... it would have been obvious to one having ordinary skill in the art at the time the invention was made, to use aspartyl moiety in linker 1 of the affinity ligand because (1) Rosebrough teaches that by introducing an alpha carboxylate group to the amide bond in linker 1 increases stability... ." However, there was no data available at the time of the invention that provided substantiated evidence for structural features that block biotinidase. The data from the Rosebrough paper suggested that an alpha-carboxylate might block biotinidase, but the extent of blockage without the deferoxamine was not determined. That lack of knowledge coupled with the fact that incomplete biotinidase blockage was obtained in the Rosebrough studies, made it imperative to conduct experiments to determine the effect that various modifications of biotin had on biotinidase blocking. Thus, an investigation was undertaken by Applicants to determine the effect of various structural features on blocking biotinidase. Most importantly, for biomedical applications blockage of biotinidase must be

accomplished without significantly decreasing the binding affinity of biotin with avidin or streptavidin. While in the Rosebrough paper it is stated that the biotin-cysteine-deferoxamine derivative (DACB) maintained its ability to bind avidin in vitro and in vivo, the binding affinity relative to native biotin was not determined. Biotin derivatives that have low binding affinities maintain their ability to bind with avidin, but they are not useful in the intended biomedical application. Thus, the investigation of a number of structurally different biotin derivatives began with evaluating their relative dissociation rates from avidin and streptavidin (*Bioconjugate Chemistry 11*, 569-583, 2000). Biotin derivatives that retained high binding affinities were subsequently examined for biotinidase cleavage (*Bioconjugate Chem. 12*, 616-623, 2001). The first substantiated data that indicated an aspartyl group alpha to the biotin moiety completely blocked biotinidase activity and retained high binding affinity with avidin and streptavidin came from the data obtained in these studies.

Applicants submit herewith an article by Mock et al. which confirms that those of skill in the art did not take the data presented by Rosebrough as conclusive that alpha-carboxylate would block biotinidase. See Mock et al., *Analytical Biochemistry*; 337, pp. 98-102, 2005. As explained in the article, Mock "tested the hypothesis that a biotin–protein conjugate containing a carboxylate alpha to the amide bond would be stable in plasma (emphasis added)." Hence, Applicants submit, the above statement of Mock is a perfect example of how one of ordinary skill who, while he (Mock) had read Rosebrough's and Applicants' papers on biotinidase, still felt that this needed to be tested as a conjugate before it could be said with certainty that it would be stable.

In the Office Action, it was also stated that "... it would have been obvious to one having ordinary skill in the art at the time the invention was made, to use aspartyl moiety in linker 1 of the affinity ligand because ... (2) a homologous series is a family of chemically related compounds, the composition varies from member to member by

CH2***, wherein Chemists knowing the properties of one member would in general know what to expect in adjacent members... ." This is a true statement when discussing spectral properties of a molecule, but it does not apply to expectations for the molecules in biological systems. It is not possible to predict or "know what to expect" for modifications of biomolecules such as biotin, which bind to receptors and are metabolized by specific enzymes. Structure-activity analyses are conducted to allow prediction of the interaction for biological molecules with receptors and enzymes. Structure-activity analyses are based on preparing many different derivatives of a molecule. One can not predict the bioactivity of an enzyme such as biotinidase on a biotin derivative based on a single example of an alpha-carboxylate, nor can one predict how such a carboxylate will affect the binding affinity with avidin or streptavidin without preparing a series of compounds to probe the interaction. Molecular modeling can assist in such predictions, but this requires a crystal structure of the biomolecule interactions to provide the three-dimensional model. While crystal structures of avidin and streptavidin are available, this tool does not at present provide adequate assurance that values obtained are correct, so laboratory experiments still have to be conducted. Hence, one of ordinary skill in the art would not necessarily have expected the results obtained when using an alpha carboxylate to correlate to an aspartyl moiety

In view of the above remarks, this rejection is overcome and its withdrawal is requested.

* * *

Claims 36 and 39 are rejected under 35 U.S.C. § 103(a) as purportedly obvious based on Wilbur et al. in view of Rosebrough, and further in view of Griffiths (U.S. Pat. No. 5,482,698). Applicants again respectfully traverse.

The deficiencies of Wilbur and Rosebrough are discussed above. Moreover, Griffiths fails to remedy these deficiencies. None of the cited documents teach or fairly

enzymatic cleavage. Moreover, there is nothing in the combined teachings of the cited documents which would have motivated one of ordinary skill in the art to do so.

In view of the above remarks, Applicants respectfully submit that this rejection is overcome. Reconsideration and withdrawal of the rejection is thus requested.

* * *

Claims 33, 34, 36, 38, 40, 41, 55-59, 62, 64-67, 70, 73, 74, 88, and 89 are provisionally rejected under the doctrine of obviousness-type double patenting as obvious based on the claims of co-pending application 09/519,998. Applicants also respectfully traverse this rejection.

The Office Action conceded that the '998 application does not disclose the aspartyl moiety. Hence, Applicants again refer to the above discussion with respect whether the use of an alpha carboxylate renders the use of an aspartyl moiety obvious. Moreover, Applicants again submit, one can not predict the bioactivity of an enzyme such as biotinidase on a biotin derivative based on a single example of an alpha-carboxylate, nor can one predict how such a carboxylate will affect the binding affinity with avidin or streptavidin without preparing a series of compounds to probe the interaction. Hence, there is nothing in the '998 application which would have motivated one of ordinary skill in the art to employ an aspartyl moiety to stabilize a biotinamide bonds against enzymatic degradation. Withdrawal of this rejection is thus requested.

* * * * *

Applicants respectfully submit that this Amendment and the above remarks obviate the outstanding rejections in this case, thereby placing the application in condition for immediate allowance. Allowance of this application is earnestly solicited.

If any fees under 37 C.F.R. §§ 1.16 or 1.17 are due in connection with this filing, please charge the fees to Deposit Account No. 02-4300; Order No. 033700.005.

If an extension of time under 37 C.F.R. § 1.136 is necessary that is not accounted for in the papers filed herewith, such an extension is requested. The extension fee should be charged to Deposit Account No. 02-4300; Order No. 033700.005.

Respectfully submitted,

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A biotin–protein bond with stability in plasma

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Abstract

A nonradioactive label for peptide hormones would be useful for pharmacokinetic studies in infants, children, and pregnant women. Because the binding affinity between biotin and avidin is large ($K_a = 10^{15}\,M^{-1}$), biotin could also serve as a covalent label for subsequent detection using a variety of avidin conjugates. However, biotin labels produced by most commercially available biotinylating reagents are rapidly cleaved from protein in plasma. We sought to synthesize a stable biotin label for protein. With the use of immunoglobulin G (IgG) as a model protein, biotin was conjugated through a cysteine residue; a carboxylate group was positioned alpha to the biotinamide bond. Stability of the bond in the presence of plasma and buffer control was assessed by release of biotin. Released biotin was separated from biotinylated IgG by ultrafiltration and was quantitated by an avidin-binding assay. In plasma, less than 0.6% of bound biotin was released. This release rate is not significantly different from buffer and is less than 7% of the release rate for IgG biotinylated by *N*-hydroxysuccinimide-LC-biotin. We conclude that this biotin–protein bond is stable in plasma. We speculate that many uses of avidin–biotin technology could be improved by using this method for protein labeling.

Keywords: Biotin; Biotinylation; Avidin; Immunoglobulin G

We are investigating the use of biotin-labeled proteins in determining pharmacokinetics in humans. A design criterion is that the biotin label must be stable in plasma for approximately 48 h. However, our studies of the stability of the bond between biotin and the model protein immunoglobulin G (IgG)[1] indicate that biotin bonds produced by most commercial biotinylating agents are not stable in plasma when assessed after only 4 h of exposure [1]. A similar instability of biotin conjugates used in radioimaging and radionuclide targeting of tumors has been reported [2,3]. We report here an adaptation of an approach reported by Rosebrough [3,4] to synthesize a biotin bound to protein that is stable in plasma.

Materials and methods

Materials

All chemicals and reagents were analytical grade. Ultrapure deionized water (Millipore, Bedford, MA, USA) was used throughout. Rabbit IgG, avidin, dimethyl sulfoxide (Me_2SO), polyoxyethylenesorbitan monolaureate (Tween 20), iodoacetic acid *N*-hydroxysuccinimide ester (iodoacetic acid-NHS), and Dulbecco's phosphate-buffered saline (PBS) were obtained from Sigma–Aldrich Chemical (St. Louis, MO, USA). NHS-biotin, L-cysteine, and BCA reagent were purchased from Pierce (Rockford, IL, USA).

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[1] *Abbreviations used:* IgG, immunoglobulin G; Me_2SO, dimethyl sulfoxide; Tween 20, polyoxyethylenesorbitan monolaureate; iodoacetic acid-NHS, iodoacetic acid *N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; Bio-C-A-IgG, biotinyl-cysteinyl-acetyl-IgG; BC, biotinyl-cysteine; MALDI, matrix-assisted laser desorption/ionization; IA-IgG, iodo-acetyl-IgG; BH, biotin-LC-hydrazide.

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Methods

Synthesis of biotinyl-cysteinyl-acetyl-IgG

The synthesis of biotinyl-cysteinyl-acetyl-IgG (Bio-C-A-IgG) required the formation of two intermediate compounds: biotinyl-cysteine and iodo-acetyl-IgG (Fig. 1).

Biotinyl-cysteine. Biotinyl-cysteine (BC) was synthesized by a modification of the published procedure [3,4]. A solution of L-cysteine (37.6 μmol) in 0.1 M NaH_2PO_4 (1 ml) was mixed with NHS-biotin in 1 ml of 70% methanol in water (18.7 μmol). The pH of the reaction mixture was adjusted to 8.0 and was agitated at room temperature for 1 h. The reaction mixture was adjusted to pH 2.0 with HCl and was kept overnight at 4 °C. The resulting crystalline biotinyl-cysteine was washed three times with water, and crystals were then dissolved in 500 μl of 70% methanol in 0.1 M NaH_2PO_4. Hashmi and Rosebrough [4] reported the structure of this compound using ^1H NMR and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

Iodo-acetyl-IgG. A solution of IgG (13.4 nmol) in PBS (1 ml, pH 7.2) was mixed with 76 μl of iodoacetic acid-NHS (270 nmol) in Me_2SO. The reaction mixture was agitated at room temperature for 1 h. Tween 20 was added (0.05 mM final concentration), and iodo-acetyl-IgG (IA-IgG) was separated from low-molecular weight reactants by dialysis against PBS with Tween 20 in a 10-kDa cutoff device (Slidalyzer, Pierce).

Biotinyl-cysteinyl-acetyl-IgG. A dialyzed solution of IA-IgG (~1.3 ml) was mixed with 100 μl of biotinyl-cysteine in 70% methanol in 0.1 M NaH_2PO_4. The pH of the reaction mixture was adjusted to 8.2 and was agitated at room temperature overnight. Low-molecular weight reactants were removed by dialysis as described above.

Determination of the stoichiometry of biotinylation for Bio-C-A-IgG

Protein concentrations were determined by BCA protein assay (Pierce). Total biotin covalently bound to IgG was determined by releasing biotin by acid hydrolysis, and released biotin was quantitated using an avidin-binding assay as described previously [5,6].

Human plasma

Blood was collected from healthy adults using heparin as the anticoagulant. Plasma was harvested by centrifugation at 3000*g* for 10 min. The plasma from four individuals was pooled for these studies. The institutional review

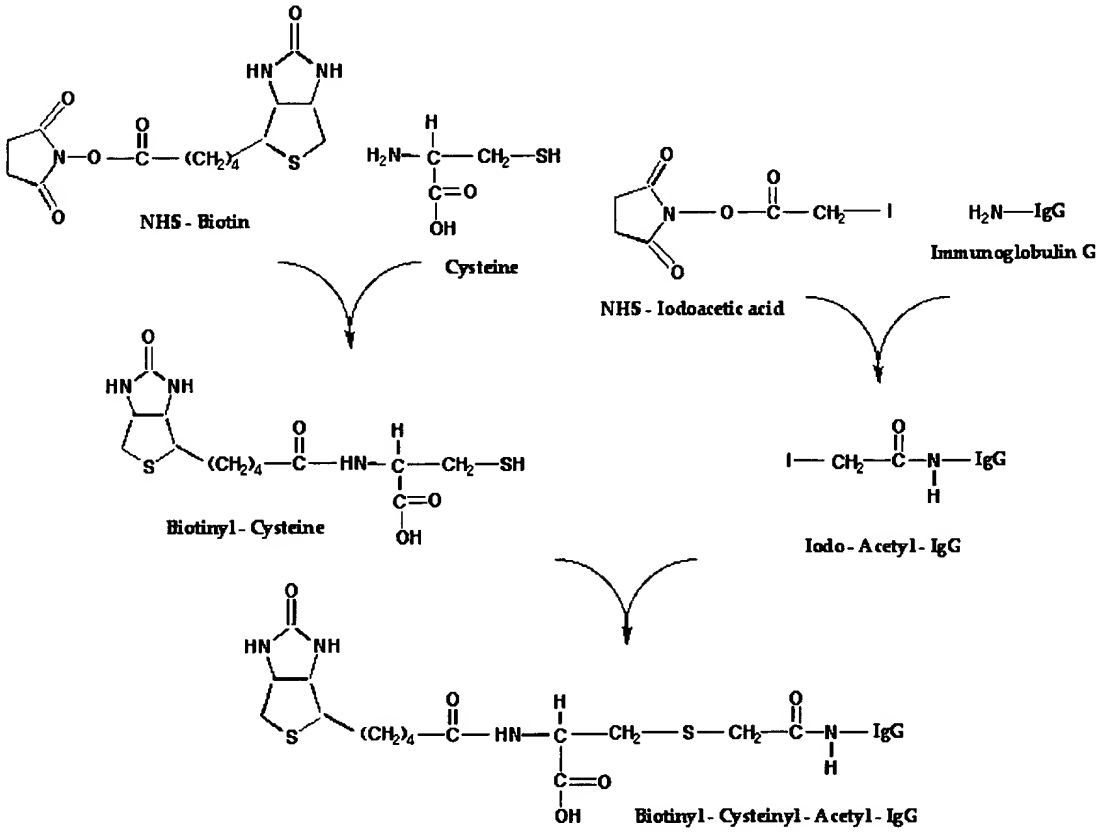

Fig. 1. Reaction scheme for the synthesis of Bio-C-A-IgG.

board of the University of Arkansas for Medical Sciences approved this study.

Removal of free biotin from human plasma

To accurately quantitate biotin released by human plasma, all free biotin must be removed from the plasma prior to use in stability studies. Human plasma was dialyzed extensively against PBS buffer containing avidin with 2.5 times more binding capacity for biotin than the estimated biotin content of the plasma (the normal range is 294–1021 pM). After dialysis, the free biotin concentration in the "biotin-free plasma" was undetectable ($\leqslant 22$ pM). Biotin-free plasma was stored at −20 °C.

Quantitation of the Bio-C-A-IgG bond stability

The stability of the biotin–protein bond was assessed by measuring biotin release. Biotinylated IgG was incubated at room temperature for 4 h in PBS buffer (control) or biotin-free plasma. After 1:1 dilution with water, 2 ml of the incubation mixture was ultrafiltered with a Centricon 10-kDa device (Millipore). Biotin released from the protein was quantitated in the ultrafiltrate using an avidin-binding assay as described previously [6].

Statistical methods

Differences between groups (e.g., plasma vs. buffer) were tested using the unpaired t test and were considered significant at $P < 0.05$. Data are expressed as means ± 1 standard deviation (SD).

Results

The synthetic scheme for the preparation of Bio-C-A-IgG is depicted in Fig. 1. The final linkage between biotin and the ε-amino groups of the lysine residues in IgG contains a carboxyl group attached to the carbon that is alpha to the biotinamide bond. The first synthetic step is the synthesis of biotinyl-cysteine. Biotinyl-cysteine was formed by the aminolysis of NHS biotin by L-cysteine as described in Materials and methods. IA-IgG was synthesized via the nucleophilic reaction of IgG with iodoacetic acid-NHS. Bio-C-A-IgG was prepared by the conjugation reaction of biotinyl-cysteine and iodo-acetyl-IgG; in this reaction, iodine from iodo-acetyl-IgG was displaced by the thiol of biotinyl-cysteine (Fig. 1). Overall yield of Bio-C-A-IgG from IgG was 60% as quantitated by protein assay.

The total number of bound biotin moieties was quantitated as release of biotin by acid hydrolysis of Bio-C-A-IgG. The stoichiometry (mole biotin:mole IgG) was 3.6. This stoichiometry is similar to that achieved by other biotinylating agents. For the agents listed in Table 1, stoichiometries ranged from 0.2 to 4.5 [1].

The stability of the biotinylated IgG was determined by quantitation of the percentage of total biotin released after 4 h of incubation with plasma or PBS buffer control. The new synthetic scheme produced biotinylated IgG that was stable in both buffer and plasma. Only 0.53% of the total biotin label was released by incubation in plasma (Fig. 2); this rate is not significantly different from control. We had previously investigated the stability of the biotin bond produced by common biotinylation reagents that are commercially available [1]. These results are provided here (Fig. 2) for comparison. The amide bond formed by commercially available biotinylation reagents is 13–143 times more susceptible to plasma cleavage than is Bio-C-A-IgG (Fig. 2). Rates of cleavage in buffer are not significantly different except for the hydrazone bond produced by biotin-LC-hydrazide (BH). For BH, the rate of hydrolysis in buffer is 78 times that for Bio-C-A-IgG.

Table 1
Stoichiometry of biotinylation of IgG

Biotinylation reagent	Biotin/protein (mol/mol)
Biotin-PEO-amine	0.3
5-(Biotinamido)-pentylamine	0.2
Iodoacetyl-LC-biotin	2.7
Biotin-LC-hydrazide	0.4
Sulfo-NHS-LC-biotin	4.3
NHS-LC-biotin	4.5
Biotinyl-cysteine	3.6

Note. Degree of biotinylation was determined by avidin-binding assay of biotinylated IgG after acid hydrolysis to release covalently bound biotin.

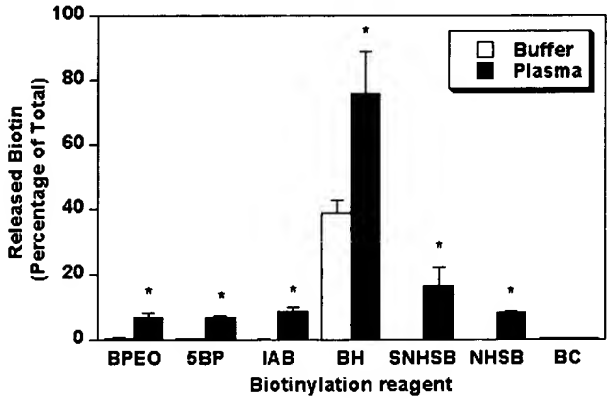

Fig. 2. Biotin released expressed as percentage of total biotin. IgG was biotinylated with a variety of biotinylation reagents—biotin-PEO-amine (BPEO), 5-(biotinamido)pentylamine (5BP), iodoacetyl-LC-biotin (IAB), biotin-LC-hydrazide (BH), sulfo-NHS-LC-biotin (SNHSB), NHS-LC-biotin (NHSB), and biotinyl-cysteine (BC)—producing different spacer arms and different types of bonds to several different moieties on IgG [1]. Biotin release was quantitated after 4 h incubation with PBS buffer (control) or human plasma. Released biotin was detected by avidin-binding assay of the 10-kDa ultrafiltrate. Replicate assays are depicted as means ± SD ($n \geqslant 5$). The Bio-C-A-IgG bond is stable in plasma, but other bonds are not. *Significantly different from control ($P < 0.0001$).

Discussion

We are investigating the use of biotin-labeled proteins for pharmacokinetic studies in human subjects. Investigations in our laboratory [1,7] and in other laboratories [2,3,8–10] provide evidence that biotin bonds produced by common, commercially available biotinylation reagents are not stable in plasma. In many of these reports, biotinidase is inferred, although not directly shown, to catalyze the cleavage. Mechanistic studies from our laboratory indicate that both enzymatic and nonenzymatic factors present in plasma are capable of catalyzing the cleavage of the biotin bond [7]. Free biotin is the primary product released when biotin is cleaved from protein in plasma [7]. This finding provides evidence that the primary site of cleavage is the amide bond between the carboxyl group of biotin and the amino group on the spacer arm (if a spacer arm is present) or the ε-amino group of a lysine residue on the protein (if no spacer arm is present) [7].

Wilbur and colleagues [2,9,11–13] published a series of articles describing modifications of this amide bond by introducing steric hindrance near the amide bond to protect bond stability. Foulon et al. [8] evaluated two biotin conjugates in which the amide bond between the valeric acid side chain of biotin and the prosthetic group of a small molecule (e.g., 3-iodobenzoate) is reversed (i.e., NH–CO bond); both were stable in serum. Rosebrough's study [3] indicated that an α-carboxylate in deferoxamine–biotin conjugate could block cleavage in plasma. This conjugate contained a cysteine linker with a carboxylate alpha to the biotinamide bond.

We tested the hypothesis that a biotin–protein conjugate containing a carboxylate alpha to the amide bond would be stable in plasma. Our study provides evidence that the biotin label produced as described is stable in plasma in vitro. Loss of biotin was not significantly different from that in buffer. We speculate that biotin labels produced in this fashion will be sufficiently stable that release rates in vivo in human subjects and animal models will be negligible with respect to the rates of removal of most plasma proteins of interest. In particular, we speculate that cleavage from most peptide hormones will be negligible with respect to their rate of disappearance from plasma in vivo.

The in vitro stability of Bio-C-A-IgG in the presence of PBS buffer and human plasma indicates that Bio-C-A-IgG remains intact in both buffer and plasma. We infer that the substituent carboxylate group alpha to the biotinamide bond sterically hinders the enzymatic cleavage of biotinylated protein.

However, steric hindrance is most effective as a mechanism for blocking enzymatic cleavage. Given that approximately half of the cleavage of biotin label occurs by a nonenzymatic method, we infer that placement of a carboxyl alpha to the biotinamide bond also confers increased chemical stability toward whatever nonenzymatic factors catalyze bond hydrolysis.

Stoichiometry of biotin to IgG was 3.6:1, indicating that conjugation reactions occurred with an average of four amino groups on IgG. Our studies of the biotinylation of histones using sulfo-NHS-biotin to biotinylate poly-lysine and poly-arginine indicated a 10,000-fold preference for biotinylating lysine vs. arginine residues [14]. Given that the attacking moiety for attachment of the iodoacetic group was also NHS (iodoacetic acid-NHS), we speculate that ε-amino groups of lysine are also likely to be the biotinylated residues using the method described here.

In conclusion, we have described the synthesis and characterization of a biotinylated IgG in which biotin is linked to the protein via a cysteine linker. We found that this type of biotin–protein bond is resistant to both enzymatic and nonenzymatic plasma cleavage and may offer significant advantages for nonradioactive labeling strategies.

Acknowledgments

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